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(54) Title: REGULATION OF HUMAN HISTAMINE H2-LIKE G PROTEIN-COUPLED RECEPTOR

(57) Abstract: Reagents which regulate human histamine H2-like G protein-coupled receptor (histamine H2-like GPCR) and reagents which bind to human histamine H2-like GPCR gene products can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, those of the digestive, immune, respiratory, reproductive, urinary peripheral or central nervous systems.

## **REGULATION OF HUMAN HISTAMINE H2-LIKE G PROTEIN-COUPLED RECEPTOR**

### **TECHNICAL FIELD OF THE INVENTION**

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The invention relates to the area of G-protein coupled receptors. More particularly, it relates to the area of human histamine H2-like G protein-coupled receptor and its regulation.

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### **BACKGROUND OF THE INVENTION**

#### **G-Protein Coupled Receptors**

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Many medically significant biological processes are mediated by signal transduction pathways that involve G-proteins (Lefkowitz, *Nature* 351, 353-354, 1991). The family of G-protein coupled receptors (GPCR) includes receptors for hormones, neurotransmitters, growth factors, and viruses. Specific examples of GPCRs include receptors for such diverse agents as dopamine, calcitonin, adrenergic hormones, endothelin, cAMP, adenosine, acetylcholine, histamine H2, histamine, thrombin, kinin, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorants, cytomegalovirus, G-proteins themselves, effector proteins such as phospholipase C, adenylyl cyclase, and phosphodiesterase, and actuator proteins such as protein kinase A and protein kinase C.

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GPCRs possess seven conserved membrane-spanning domains connecting at least eight divergent hydrophilic loops. GPCRs (also known as 7TM receptors) have been characterized as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. Most GPCRs have single conserved cysteine residues in each of the first two extracellular loops, which form disulfide bonds that are believed to stabilize functional protein

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structure. The seven transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 has been implicated in signal transduction.

5 Phosphorylation and lipidation (palmitylation or farnesylation) of cysteine residues can influence signal transduction of some GPCRs. Most GPCRs contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several GPCRs, such as the  $\beta$ -adrenergic receptor, phosphorylation by protein kinase A and/or specific receptor kinases mediates receptor desensitization.

10 For some receptors, the ligand binding sites of GPCRs are believed to comprise hydrophilic sockets formed by several GPCR transmembrane domains. The hydrophilic sockets are surrounded by hydrophobic residues of the GPCRs. The hydrophilic side of each GPCR transmembrane helix is postulated to face inward and form a polar ligand binding site. TM3 has been implicated in several GPCRs as having a  
15 ligand binding site, such as the TM3 aspartate residue. TM5 serines, a TM6 asparagine, and TM6 or TM7 phenylalanines or tyrosines also are implicated in ligand binding.

GPCRs are coupled inside the cell by heterotrimeric G-proteins to various  
20 intracellular enzymes, ion channels, and transporters (*see Johnson et al., Endoc. Rev. 10, 317-331, 1989*). Different G-protein alpha-subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of GPCRs is an important mechanism for the regulation of some GPCRs. For example, in one form of signal transduction, the  
25 effect of hormone binding is the activation inside the cell of the enzyme, adenylate cyclase. Enzyme activation by hormones is dependent on the presence of the nucleotide GTP. GTP also influences hormone binding. A G-protein connects the hormone receptor to adenylate cyclase. G-protein exchanges GTP for bound GDP when activated by a hormone receptor. The GTP-carrying form then binds to  
30 activated adenylate cyclase. Hydrolysis of GTP to GDP, catalyzed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G-protein serves a

- 3 -

dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the duration of the signal.

5 Over the past 15 years, nearly 350 therapeutic agents targeting GPCRs receptors have been successfully introduced onto the market. This indicates that these receptors have an established, proven history as therapeutic targets. Clearly, there is an on-going need for identification and characterization of further GPCRs which can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, infections such as bacterial, fungal, protozoan, and viral infections, 10 particularly those caused by HIV viruses, cancers, anorexia, bulimia, acute heart failure, hypotension, hypertension, urinary retention, osteoporosis, angina pectoris, myocardial infarction, ulcers, asthma, allergies, benign prostatic hypertrophy. GPCRs are of critical importance to both central and peripheral nervous system and novel GPCRs are therefore promising new targets for the treatment of nervous 15 system disease, for example in primary and secondary disorders after brain injury, disorders of mood, anxiety disorders, disorders of thought and volition, disorders of sleep and wakefulness, diseases of the motor unit like neurogenic and myopathic disorders, neurodegenerative disorders like Alzheimer's and Parkinson's disease, disorders leading to peripheral and chronic pain.

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Because of the wide-spread distribution of GPCRs with diverse biological effects, there is a need in the art to identify additional members of the GPCR family whose activity can be regulated to provide therapeutic effects.

### 25 Histamine and its Receptors

Histamine is found in mast cells in all peripheral tissues. It is released via degranulation of mast cells as part of allergic and inflammatory responses to allergens, infection or trauma. Chronic inflammation can contribute to degradation 30 of host tissue and will eventually lead to tissue destruction and to clinical disease associated with loss of organ function. Histamine is widely distributed in both

neuronal and non-neuronal cells and has been implicated in a variety of CNS functions including arousal and analgesia.

5 In low concentrations, histamine mediates inflammation and allergies through the activation of the H1 receptor. The H1 receptor is widely distributed, regulates the contraction of smooth muscles of the intestine, trachea, bladder and blood vessels. The H1 receptor is also associated with the adrenal medulla, vascular endothelium, heart, cerebral cortex, cerebellum and spinal cord. In high concentrations, histamine regulates the release of gastric acid from parietal cells of the digestive system  
10 through activation of the histamine H2 receptor. The H2 receptor is found at high levels in heart and stomach and has limited distribution in smooth muscle, the uterus, and cells of the immune system. Both the H1 and the H2 receptors are seven transmembrane, G-protein coupled receptors. See U.S. Patents 5,817,480 and 5,885,824.

15 Much of the known information on histamine receptors, their conformations and activities is based on observations made following the administration of antagonists. For example, conventional antihistamines block the actions of histamine on smooth muscle of blood vessels, gut, or bronchi, but they do not inhibit histamine-stimulated  
20 gastric acid secretion. This observation led to further study of histamine receptors and the development of specific classes of antagonists for the different types of receptors. The conventional histamine receptor which is blocked by classic anti-histamines is the known H-1 receptor whereas the gastric parietal cell or H-2 receptor (H2R) is not affected. H1 antagonists are used clinically in the treatment of allergic,  
25 asthmatic and anaphylactic reactions. The H2R antagonists are potent inhibitors of gastric acid secretion, exhibit some structural similarities to histamine and to each other, and are generally effective in healing and reducing the occurrence of gastric ulcers, particularly duodenal ulcers.

30 Perhaps the best known activity of histamine involves its relationship to gastric acid secretion via the H2 receptor. Antagonism of the histamine ligand at the H2 receptor

has been the cornerstone of a current \$3 billion market for pharmacological treatment of acid-peptic disorders of the gastrointestinal tract. Because of the variety of actions mediated by members of the histamine receptor family, there is a need in the art to identify additional histamine receptors whose activity can be modulated to provide therapeutic effects.

### **SUMMARY OF THE INVENTION**

It is an object of the invention to provide reagents and methods of regulating a human histamine H<sub>2</sub>-like G protein-coupled receptor 1 protein. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention is a histamine H<sub>2</sub>-like GPCR polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 50% identical to the amino acid sequence shown in SEQ ID NO: 2; and

the amino acid sequence shown in SEQ ID NO: 2.

Yet another embodiment of the invention is a method of screening for agents which decrease the activity of histamine H<sub>2</sub>-like GPCR. A test compound is contacted with a histamine H<sub>2</sub>-like GPCR polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 50% identical to the amino acid sequence shown in SEQ ID NO: 2; and

the amino acid sequence shown in SEQ ID NO: 2.

Binding between the test compound and the histamine H2-like GPCR polypeptide is detected. A test compound which binds to the histamine H2-like GPCR polypeptide is thereby identified as a potential agent for decreasing the activity of histamine H2-like GPCR.

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Another embodiment of the invention is a method of screening for agents which decrease the activity of histamine H2-like GPCR. A test compound is contacted with a polynucleotide encoding a histamine H2-like GPCR polypeptide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

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nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1; and

15

the nucleotide sequence shown in SEQ ID NO: 1.

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Binding of the test compound to the polynucleotide is detected. A test compound which binds to the polynucleotide is identified as a potential agent for decreasing the activity of histamine H2-like GPCR. The agent can work by decreasing the amount of the histamine H2-like GPCR through interacting with the histamine H2-like GPCR mRNA.

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Another embodiment of the invention is a method of screening for agents which regulate the activity of histamine H2-like GPCR. A test compound is contacted with a histamine H2-like GPCR polypeptide comprising an amino acid sequence selected from the group consisting of:

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amino acid sequences which are at least about 50% identical to the amino acid sequence shown in SEQ ID NO: 2; and

the amino acid sequence shown in SEQ ID NO: 2.

A histamine H2-like GPCR activity of the polypeptide is detected. A test compound which increases histamine H2-like GPCR activity of the polypeptide relative to histamine H2-like GPCR activity in the absence of the test compound is thereby identified as a potential agent for increasing the activity of histamine H2-like GPCR. A test compound which decreases histamine H2-like GPCR activity of the polypeptide relative to histamine H2-like GPCR activity in the absence of the test compound is thereby identified as a potential agent for decreasing the activity of histamine H2-like GPCR.

Even another embodiment of the invention is a method of screening for agents which decrease the activity of histamine H2-like GPCR. A test compound is contacted with a histamine H2-like GPCR product of a polynucleotide which comprises a nucleotide sequence selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1; and

the nucleotide sequence shown in SEQ ID NO: 1.

Binding of the test compound to the histamine H2-like GPCR product is detected. A test compound which binds to the histamine H2-like GPCR product is thereby identified as a potential agent for decreasing the activity of histamine H2-like GPCR.

Still another embodiment of the invention is a method of reducing the activity of histamine H2-like GPCR. A cell is contacted with a reagent which specifically binds to a polynucleotide encoding a histamine H2-like GPCR polypeptide or the product encoded by the polynucleotide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:



nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1; and

the nucleotide sequence shown in SEQ ID NO: 1.

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Histamine H2-like GPCR activity in the cell is thereby decreased.

The invention thus provides a human histamine H2-like G protein-coupled receptor which can be used to identify test compounds which may act as agonists or  
10 antagonists at the receptor site. Human histamine H2-like G protein-coupled receptor and fragments thereof also are useful in raising specific antibodies which can block the receptor and effectively prevent ligand binding.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

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Fig. 1 shows the DNA-sequence encoding histamine H2-like GPCR polypeptide.

Fig. 2 shows the amino acid sequence deduced from the DNA-sequence of Fig.1.

20 Fig. 3 shows the results from Taqman expression profiling of the H2-like GPCR in human brain tissue.

Fig. 4 shows a diagram of the expression profiling results from Fig. 3

### **DETAILED DESCRIPTION OF THE INVENTION**

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The invention relates to an isolated polynucleotide encoding a histamine H2-like GPCR polypeptide and being selected from the group consisting of:

- 30 a) a polynucleotide encoding histamine H2-like GPCR polypeptide comprising an amino acid sequence selected from the group consisting of:  
amino acid sequences which are at least about 50% identical to

the amino acid sequence shown in SEQ ID NO: 2; and  
the amino acid sequence shown in SEQ ID NO: 2.

- b) a polynucleotide comprising the sequence of SEQ ID NO: 1;
- c) a polynucleotide which hybridizes under stringent conditions to a  
5 polynucleotide specified in (a) and (b);
- d) a polynucleotide the sequence of which deviates from the polynucleotide  
sequences specified in (a) to (c) due to the degeneration of the genetic code;  
and
- e) a polynucleotide which represents a fragment, derivative or allelic variation of  
10 a polynucleotide sequence specified in (a) to (d).

Furthermore, it has been discovered by the present applicant, that a histamine H2-like  
G protein-coupled receptor (histamine H2-like GPCR) protein, particularly human  
histamine H2-like GPCR protein, can be used in therapeutic methods to treat  
15 disorders such as anxiety, depression, hypertension, migraine, compulsive disorders,  
schizophrenia, autism, neurodegenerative disorders, such as Alzheimer's disease,  
Parkinsonism, and Huntington's chorea, and cancer chemotherapy-induced vomiting.  
Human histamine H2-like GPCR also can be used to screen for histamine H2-like  
GPCR agonists and antagonists.

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#### Histamine H2-like GPCR Polypeptides

Histamine H2-like GPCR polypeptides according to the invention comprise the  
amino acid sequence shown in SEQ ID NO:2, a portion of that sequence, or a  
25 biologically active variant thereof, as defined below. A histamine H2-like GPCR  
polypeptide of the invention therefore can be a portion of a histamine H2-like GPCR  
protein, a full-length histamine H2-like GPCR protein, or a fusion protein comprising  
all or a portion of a histamine H2-like GPCR protein. A coding sequence for SEQ  
ID NO:2 is shown in SEQ ID NO:1. The DNA sequence of the receptor provided by  
30 the present invention can be used in the development of compounds useful for the  
treatment of a wide variety of pathologic states in which histamine is involved. For

example, the usefulness of H2 blockers in the treatment of acid peptic disorders is well known. However, despite the great selectivity of the H2 blockers presently are clinical, all are known to have some undesirable side effects. Development of even more selective antagonists can stem from a more complete understanding of the molecular structure of the receptor provided by the present invention.

#### Biologically Active Variants

Histamine H2-like GPCR polypeptide variants which are biologically active, *i.e.*, retain the ability to bind histamine H2 or a histamine-like ligand to produce a biological effect, such as cyclic AMP formation, mobilization of intracellular calcium, or phosphoinositide metabolism, also are histamine H2-like GPCR polypeptides. Preferably, naturally or non-naturally occurring histamine H2-like GPCR polypeptide variants have amino acid sequences which are at least about 50, preferably about 75, 90, 96, or 98% identical to the amino acid sequence shown in SEQ ID NO:2 or a fragment thereof. Percent identity between a putative histamine H2-like GPCR polypeptide variant and an amino acid sequence of SEQ ID NO:2 is determined using the Blast2 alignment program.

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a histamine H2-like GPCR polypeptide can be found using computer programs well known in the art, such as DNASTAR

software. Whether an amino acid change results in a biologically active histamine H2-like GPCR polypeptide can readily be determined by assaying for binding to a ligand or by conducting a functional assay, as described for example, in the specific Examples, below.

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### Fusion Proteins

Fusion proteins can comprise at least 5, 6, 8, 10, 25, or 50 or more contiguous amino acids of an amino acid sequence shown in SEQ ID NO:2. Fusion proteins are useful for generating antibodies against histamine H2-like GPCR polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions of a histamine H2-like GPCR polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A histamine H2-like GPCR polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises at least 5, 6, 8, 10, 25, or 50 or more contiguous amino acids of SEQ ID NO:2 or from a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length histamine H2-like GPCR protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include  $\beta$ -galactosidase,  $\beta$ -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose

binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the histamine H2-like GPCR polypeptide-encoding sequence and the heterologous protein sequence, so that the histamine H2-like GPCR polypeptide can be cleaved and purified away from the heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO:1 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

20

#### Identification of Species Homologs

Species homologs of human histamine H2-like GPCR polypeptide can be obtained using histamine H2-like GPCR polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of histamine H2-like GPCR polypeptide, and expressing the cDNAs as is known in the art.

### Histamine H2-like GPCR Polynucleotides

A histamine H2-like GPCR polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a histamine  
5 H2-like GPCR polypeptide. A coding sequence for human histamine H2-like GPCR is shown in SEQ ID NO:1.

Degenerate nucleotide sequences encoding human histamine H2-like GPCR polypeptides, as well as homologous nucleotide sequences which are at least about  
10 50, preferably about 75, 90, 96, or 98% identical to the nucleotide sequence shown in SEQ ID NO:1 also are histamine H2-like GPCR polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2.  
15 Complementary DNA (cDNA) molecules, species homologs, and variants of histamine H2-like GPCR polynucleotides which encode biologically active histamine H2-like GPCR polypeptides also are histamine H2-like GPCR polynucleotides.

### Identification of Variants and Homologs of Histamine H2-like GPCR Polynucleotides

  
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Variants and homologs of the histamine H2-like GPCR polynucleotides described above also are histamine H2-like GPCR polynucleotides. Typically, homologous histamine H2-like GPCR polynucleotide sequences can be identified by  
25 hybridization of candidate polynucleotides to known histamine H2-like GPCR polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50  $\mu$ C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes  
30 each--homologous sequences can be identified which contain at most about 25-30%

basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the histamine H2-like GPCR polynucleotides disclosed herein  
5 also can be identified by making suitable probes or primers and screening cDNA  
expression libraries from other species, such as mice, monkeys, or yeast. Human  
variants of histamine H2-like GPCR polynucleotides can be identified, for example,  
by screening human cDNA expression libraries. It is well known that the  $T_m$  of a  
double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology  
10 (Bonner *et al.*, *J. Mol. Biol.* 81, 123 (1973). Variants of human histamine H2-like  
GPCR polynucleotides or histamine H2-like GPCR polynucleotides of other species  
can therefore be identified by hybridizing a putative homologous histamine H2-like  
GPCR polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID  
NO:1 or the complement thereof to form a test hybrid. The melting temperature of  
15 the test hybrid is compared with the melting temperature of a hybrid comprising  
transformylase polynucleotides having perfectly complementary nucleotide  
sequences, and the number or percent of basepair mismatches within the test hybrid  
is calculated.

20 Nucleotide sequences which hybridize to transformylase polynucleotides or their  
complements following stringent hybridization and/or wash conditions also are  
histamine H2-like GPCR polynucleotides. Stringent wash conditions are well  
known and understood in the art and are disclosed, for example, in Sambrook *et al.*,  
MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

25 Typically, for stringent hybridization conditions a combination of temperature and  
salt concentration should be chosen that is approximately 12-20 °C below the  
calculated  $T_m$  of the hybrid under study. The  $T_m$  of a hybrid between a histamine  
H2-like GPCR polynucleotide having a nucleotide sequence shown in SEQ ID NO:1  
30 or the complement thereof and a polynucleotide sequence which is at least about 50,  
preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences

can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1390 (1962):

$$T_m = 81.5\text{ }^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\%\text{formamide}) - 600/l,$$

5 where  $l$  = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65 °C, or 50% formamide, 4X SSC at 42 °C, or 0.5X SSC, 0.1% SDS at 65 °C. Highly stringent wash conditions include, for example, 0.2X SSC at 65 °C.

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#### Preparation of histamine H2-like GPCR Polynucleotides

A naturally occurring histamine H2-like GPCR polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated histamine H2-like GPCR polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments which comprises histamine H2-like GPCR nucleotide sequences. Isolated polynucleotides are in preparations which are free or at least 70, 80, or 90% free of other molecules.

25 Histamine H2-like GPCR cDNA molecules can be made with standard molecular biology techniques, using histamine H2-like GPCR mRNA as a template. histamine H2-like GPCR cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

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Alternatively, synthetic chemistry techniques can be used to synthesize histamine H2-like GPCR polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a histamine H2-like GPCR polypeptide having, for example, an amino acid sequence shown in SEQ ID NO:2 or a biologically active variant thereof.

Extending Histamine H2-like GPCR Polynucleotides

Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, *PCR Methods Applic.* 2, 318-322, 1993). Genomic DNA is first amplified in the presence of a primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR also can be used to amplify or extend sequences using divergent primers based on a known region (Triglia *et al.*, *Nucleic Acids Res.* 16, 8186, 1988). Primers can be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72 °C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which can be used is capture PCR, which involves PCR amplification of DNA fragments adjacent to a known sequence in human and yeast

artificial chromosome DNA (Lagerstrom *et al.*, *PCR Methods Applic. 1*, 111-119, 1991). In this method, multiple restriction enzyme digestions and ligations also can be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

5

Another method which can be used to retrieve unknown sequences is that of Parker *et al.*, *Nucleic Acids Res. 19*, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

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When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Randomly-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries can be useful for extension of sequence into 5' non-transcribed regulatory regions.

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Commercially available capillary electrophoresis systems can be used to analyze the size or confirm the nucleotide sequence of PCR or sequencing products. For example, capillary sequencing can employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity can be converted to electrical signal using appropriate software (e.g. GENOTYPER and Sequence NAVIGATOR, Perkin Elmer), and the entire process from loading of samples to computer analysis and electronic data display can be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

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### Obtaining Histamine H2-like GPCR Polypeptides

Histamine H2-like GPCR polypeptides can be obtained, for example, by purification from human cells, by expression of histamine H2-like GPCR polynucleotides, or by  
5 direct chemical synthesis.

### Protein Purification

Histamine H2-like GPCR polypeptides can be purified from any human cell which  
10 expresses the receptor, including host cells which have been transfected with histamine H2-like GPCR polynucleotides. Stomach cells, notably gastric parietal cells, are particularly useful sources of histamine H2-like GPCR. A purified histamine H2-like GPCR polypeptide is separated from other compounds which normally associate with the histamine H2-like GPCR polypeptide in the cell, such as  
15 certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

20 Histamine H2-like GPCR polypeptide can be conveniently isolated as a complex with its associated G protein, as described in the specific examples, below. A preparation of purified histamine H2-like GPCR polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel  
25 electrophoresis.

### Expression of histamine H2-like GPCR Polynucleotides

To express a histamine H2-like GPCR polypeptide, a histamine H2-like GPCR  
30 polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding

sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding histamine H2-like GPCR polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a histamine H2-like GPCR polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of the vector -- enhancers, promoters, 5' and 3' untranslated regions -- which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (*e.g.*, heat shock, RUBISCO, and storage protein genes) or from plant viruses (*e.g.*, viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple

copies of a nucleotide sequence encoding a histamine H2-like GPCR polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

### Bacterial and Yeast Expression Systems

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In bacterial systems, a number of expression vectors can be selected depending upon the use intended for the histamine H2-like GPCR polypeptide. For example, when a large quantity of a histamine H2-like GPCR polypeptide is needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified can be used. Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). In a BLUESCRIPT vector, a sequence encoding the histamine H2-like GPCR polypeptide can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase so that a hybrid protein is produced. pIN vectors (Van Heeke & Schuster, *J. Biol. Chem.* 264, 5503-5509, 1989) or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

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In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH can be used. For reviews, see Ausubel *et al.* (1989) and Grant *et al.*, *Methods Enzymol.* 153, 516-544, 1987.

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### Plant and Insect Expression Systems

If plant expression vectors are used, the expression of sequences encoding histamine H2-like GPCR polypeptides can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV (Takamatsu, *EMBO J.* 6, 307-311, 1987). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters can be used (Coruzzi *et al.*, *EMBO J.* 3, 1671-1680, 1984; Broglie *et al.*, *Science* 224, 838-843, 1984; Winter *et al.*, *Results Probl. Cell Differ.* 17, 85-105, 1991). These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (*e.g.*, Hobbs or Murray, in MCGRAW HILL YEARBOOK OF SCIENCE AND TECHNOLOGY, McGraw Hill, New York, N.Y., pp. 191-196, 1992).

An insect system also can be used to express a histamine H2-like GPCR polypeptide. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding histamine H2-like GPCR polypeptides can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of histamine H2-like GPCR polypeptides will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which histamine H2-like GPCR polypeptides can be expressed (Engelhard *et al.*, *Proc. Nat. Acad. Sci.* 91, 3224-3227, 1994).

### Mammalian Expression Systems

A number of viral-based expression systems can be used to express histamine H2-like GPCR polypeptides in mammalian host cells. For example, if an adenovirus is

- used as an expression vector, sequences encoding histamine H2-like GPCR polypeptides can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing a histamine H2-like GPCR polypeptide in infected host cells (Logan & Shenk, *Proc. Natl. Acad. Sci.* 81, 3655-3659, 1984). If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can be used to increase expression in mammalian host cells.
- Human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are constructed and delivered to cells via conventional delivery methods (*e.g.*, liposomes, polycationic amino polymers, or vesicles).
- Specific initiation signals also can be used to achieve more efficient translation of sequences encoding histamine H2-like GPCR polypeptides. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding a histamine H2-like GPCR polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used (see Scharf *et al.*, *Results Probl. Cell Differ.* 20, 125-162, 1994).

### Host Cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed histamine H2-like GPCR polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high-yield production of recombinant proteins. For example, cell lines which stably express histamine H2-like GPCR polypeptides can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced histamine H2-like GPCR sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. See, for example, ANIMAL CELL CULTURE, R.I. Freshney, ed., 1986.

Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell* 11, 223-32, 1977) and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell* 22, 817-23, 1980) genes which can be employed in *tk* or *aprt* cells,



respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci.* 77, 3567-70, 1980), *npt* confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin *et al.*, *J. Mol. Biol.* 150, 1-14, 1981), and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murray, 1992, *supra*). Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci.* 85, 8047-51, 1988). Visible markers such as anthocyanins,  $\beta$ -glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes *et al.*, *Methods Mol. Biol.* 55, 121-131, 1995).

#### Detecting Expression of Histamine H2-like GPCR Polypeptides

Although the presence of marker gene expression suggests that the histamine H2-like GPCR polynucleotide is also present, its presence and expression may need to be confirmed. For example, if a sequence encoding a histamine H2-like GPCR polypeptide is inserted within a marker gene sequence, transformed cells containing sequences which encode a histamine H2-like GPCR polypeptide can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding a histamine H2-like GPCR polypeptide under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the histamine H2-like GPCR polynucleotide.

Alternatively, host cells which contain a histamine H2-like GPCR polynucleotide and which express a histamine H2-like GPCR polypeptide can be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay

or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence of a polynucleotide sequence encoding a histamine H2-like GPCR polypeptide can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding a histamine H2-like GPCR polypeptide. Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding a histamine H2-like GPCR polypeptide to detect transformants which contain a histamine H2-like GPCR polynucleotide.

A variety of protocols for detecting and measuring the expression of a histamine H2-like GPCR polypeptide, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a histamine H2-like GPCR polypeptide can be used, or a competitive binding assay can be employed. These and other assays are described in Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., 1990) and Maddox *et al.*, *J. Exp. Med.* 158, 1211-1216, 1983).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding histamine H2-like GPCR polypeptides include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a histamine H2-like GPCR polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using

a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and Purification of histamine H2-like GPCR Polypeptides

Host cells transformed with nucleotide sequences encoding a histamine H2-like GPCR polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode histamine H2-like GPCR polypeptides can be designed to contain signal sequences which direct secretion of soluble histamine H2-like GPCR polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound histamine H2-like GPCR polypeptide.

As discussed above, other constructions can be used to join a sequence encoding a histamine H2-like GPCR polypeptide to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). Inclusion of cleavable linker sequences such as those specific for Factor Xa or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the histamine H2-like GPCR polypeptide also can be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a histamine H2-like GPCR polypeptide and 6 histidine

residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by IMAC (immobilized metal ion affinity chromatography, as described in Porath *et al.*, *Prot. Exp. Purif.* 3, 263-281, 1992), while the enterokinase cleavage site provides a means for purifying the histamine  
5 H2-like GPCR polypeptide from the fusion protein. Vectors which contain fusion proteins are disclosed in Kroll *et al.*, *DNA Cell Biol.* 12, 441-453, 1993.

### Chemical Synthesis

10 Sequences encoding a histamine H2-like GPCR polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al.* *Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a histamine H2-like GPCR polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by  
15 direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85, 2149-2154, 1963; Roberge *et al.*, *Science* 269, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of histamine H2-like GPCR polypeptides can  
20 be separately synthesized and combined using chemical methods to produce a full-length molecule.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, *PROTEINS: STRUCTURES AND*  
25 *MOLECULAR PRINCIPLES*, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic histamine H2-like GPCR polypeptide can be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; see Creighton, *supra*). Additionally, any portion of the amino acid sequence of the histamine H2-like GPCR polypeptide can be altered during direct synthesis and/or  
30 combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

Production of Altered histamine H2-like GPCR Polypeptides

As will be understood by those of skill in the art, it may be advantageous to produce  
5 histamine H2-like GPCR polypeptide-encoding nucleotide sequences possessing  
non-naturally occurring codons. For example, codons preferred by a particular  
prokaryotic or eukaryotic host can be selected to increase the rate of protein  
expression or to produce an RNA transcript having desirable properties, such as a  
half-life which is longer than that of a transcript generated from the naturally  
10 occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods  
generally known in the art to alter histamine H2-like GPCR polypeptide-encoding  
sequences for a variety of reasons, including but not limited to, alterations which  
15 modify the cloning, processing, and/or expression of the polypeptide or mRNA  
product. DNA shuffling by random fragmentation and PCR reassembly of gene  
fragments and synthetic oligonucleotides can be used to engineer the nucleotide  
sequences. For example, site-directed mutagenesis can be used to insert new  
restriction sites, alter glycosylation patterns, change codon preference, produce splice  
20 variants, introduce mutations, and so forth.

Antibodies

Any type of antibody known in the art can be generated to bind specifically to an  
25 epitope of a histamine H2-like GPCR polypeptide. "Antibody" as used herein  
includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab,  
F(ab')<sub>2</sub>, and Fv, which are capable of binding an epitope of a histamine H2-like  
GPCR polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are  
required to form an epitope. However, epitopes which involve non-contiguous  
30 amino acids may require more, e.g., at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a histamine H2-like GPCR polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen.

Typically, an antibody which specifically binds to a histamine H2-like GPCR polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies which specifically bind to histamine H2-like GPCR polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a histamine H2-like GPCR polypeptide from solution.

Histamine H2-like GPCR polypeptides can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies. If desired, a histamine H2-like GPCR polypeptide can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.* lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (*bacilli Calmette-Guerin*) and *Corynebacterium parvum* are especially useful.

Monoclonal antibodies which specifically bind to a histamine H2-like GPCR polypeptide can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include,

but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler *et al.*, *Nature* 256, 495-497, 1985; Kozbor *et al.*, *J. Immunol. Methods* 81, 31-42, 1985; Cote *et al.*, *Proc. Natl. Acad. Sci.* 80, 2026-2030, 1983; Cole *et al.*, *Mol. Cell Biol.* 62, 109-120, 1984).

5

In addition, techniques developed for the production of "chimeric antibodies," the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used (Morrison *et al.*, *Proc. Natl. Acad. Sci.* 81, 6851-6855, 1984; Neuberger *et al.*, *Nature* 312, 604-608, 1984; Takeda *et al.*, *Nature* 314, 452-454, 1985). Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grating of entire complementarity determining regions. Alternatively, humanized antibodies can be produced using recombinant methods, as described in GB2188638B. Antibodies which specifically bind to a histamine H2-like GPCR polypeptide can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.

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Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which specifically bind to histamine H2-like GPCR polypeptides. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton, *Proc. Natl. Acad. Sci.* 88, 11120-23, 1991).

25

Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template (Thirion *et al.*, 1996, *Eur. J.*

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*Cancer Prev.* 5, 507-11). Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for example, in Coloma & Morrison, 1997, *Nat. Biotechnol.* 15, 159-63. Construction of bivalent, bispecific single-chain antibodies is taught in  
5 Mallender & Voss, 1994, *J. Biol. Chem.* 269, 199-206.

A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding  
10 sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology (Verhaar *et al.*, 1995, *Int. J. Cancer* 61, 497-501; Nicholls *et al.*, 1993, *J. Immunol. Meth.* 165, 81-91).

15 Antibodies which specifically bind to histamine H2-like GPCR polypeptides also can be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi *et al.*, *Proc. Natl. Acad. Sci.* 86, 3833-3837, 1989; Winter *et al.*, *Nature* 349, 293-299, 1991).

20 Other types of antibodies can be constructed and used therapeutically in methods of the invention. For example, chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO  
25 94/13804, also can be prepared.

Antibodies according to the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which a histamine H2-like GPCR polypeptide is bound. The bound antibodies can  
30 then be eluted from the column using a buffer with a high salt concentration.



### Antisense Oligonucleotides

Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of histamine H2-like GPCR gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. See Brown, *Meth. Mol. Biol.* 20, 1-8, 1994; Sonveaux, *Meth. Mol. Biol.* 26, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev.* 90, 543-583, 1990.

Modifications of histamine H2-like GPCR gene expression can be obtained by designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the histamine H2-like GPCR gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND

IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

- 5      Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a histamine H2-like GPCR polynucleotide. Antisense oligonucleotides which comprise, for example, 2, 3, 4, or 5 or more stretches of contiguous nucleotides which are precisely complementary to a histamine H2-like GPCR polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent histamine H2-like GPCR nucleotides, can provide sufficient targeting specificity for histamine H2-like GPCR mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, or 8 or more nucleotides in length. Non-complementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular histamine H2-like GPCR polynucleotide sequence.
- 10      Antisense oligonucleotides can be modified without affecting their ability to hybridize to a histamine H2-like GPCR polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. See, e.g., Agrawal *et al.*, *Trends Biotechnol.* 10, 152-158, 1992; Uhlmann *et al.*, *Chem. Rev.* 90, 543-584, 1990; Uhlmann *et al.*, *Tetrahedron. Lett.* 215, 3539-3542, 1987.
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### Ribozymes

Ribozymes are RNA molecules with catalytic activity. See, e.g., Cech, *Science* 236, 1532-1539; 1987; Cech, *Ann. Rev. Biochem.* 59, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol.* 2, 605-609; 1992, Couture & Stinchcomb, *Trends Genet.* 12, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (e.g., Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a histamine H2-like GPCR polynucleotide, such as the nucleotide sequence shown in SEQ ID NO:1, can be used to generate ribozymes which will specifically bind to mRNA transcribed from the histamine H2-like GPCR polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff *et al.* *Nature* 334, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201).

Specific ribozyme cleavage sites within a histamine H2-like GPCR RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate histamine

H2-like GPCR RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. The nucleotide sequence shown in SEQ ID NO:1 and its complement provide a source of suitable hybridization region sequences. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease histamine H2-like GPCR expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff *et al.*, U.S. Patent 5,641,673, ribozymes can be engineered so that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

#### Screening Methods

The invention provides assays for screening test compounds which bind to or modulate the activity of a histamine H2-like GPCR polypeptide or a histamine H2-like GPCR polynucleotide. A test compound preferably binds to a histamine H2-like

GPCR polypeptide or polynucleotide. More preferably, a test compound decreases or increases the effect of somatostatin or a somatostatin analog as mediated via human histamine H2-like GPCR by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

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### Test Compounds

Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. See Lam, *Anticancer Drug Des.* 12, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (see, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90, 6909, 1993; Erb *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 91, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37, 2678, 1994; Cho *et al.*, *Science* 261, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2061; Gallop *et al.*, *J. Med. Chem.* 37, 1233, 1994). Libraries of compounds can be presented in solution (see, e.g., Houghten, *Biotechniques* 13, 412-421, 1992), or on beads (Lam, *Nature* 354, 82-84, 1991), chips (Fodor, *Nature* 364, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89, 1865-1869, 1992), or phage (Scott & Smith, *Science* 249, 386-390, 1990; Devlin,

*Science* 249, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.* 97, 6378-6382, 1990; Felici, *J. Mol. Biol.* 222, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

### High Throughput Screening

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Test compounds can be screened for the ability to bind to histamine H2-like GPCR polypeptides or polynucleotides or to affect histamine H2-like GPCR activity or histamine H2-like GPCR gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500  $\mu$ l. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

15

Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 19, 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

25

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying

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combinatorial compounds via a photolinker were placed inside the gel and the compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

- 5 Yet another example is described by Salmon *et al.*, *Molecular Diversity* 2, 57-63 (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

10 Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

15

#### Binding Assays

For binding assays, the test compound is preferably a small molecule which binds to and occupies the active site of the histamine H2-like GPCR polypeptide, thereby making the ligand binding site inaccessible to substrate such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules. Potential ligands which bind to a polypeptide of the invention include, but are not limited to, the natural ligands of known histamine H2-like GPCRs and analogues or derivatives thereof.

25

In binding assays, either the test compound or the histamine H2-like GPCR polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to the histamine H2-like GPCR polypeptide can then be accomplished, for example, by

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direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a histamine H2-like GPCR polypeptide  
5 can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a histamine H2-like GPCR polypeptide. A microphysiometer (*e.g.*, Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this  
10 acidification rate can be used as an indicator of the interaction between a test compound and a histamine H2-like GPCR polypeptide (McConnell *et al.*, *Science* 257, 1906-1912, 1992).

Determining the ability of a test compound to bind to a histamine H2-like GPCR  
15 polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem.* 63, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol.* 5, 699-705, 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore™). Changes in the optical phenomenon surface  
20 plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, a histamine H2-like GPCR polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S.  
25 Patent 5,283,317; Zervos *et al.*, *Cell* 72, 223-232, 1993; Madura *et al.*, *J. Biol. Chem.* 268, 12046-12054, 1993; Bartel *et al.*, *Biotechniques* 14, 920-924, 1993; Iwabuchi *et al.*, *Oncogene* 8, 1693-1696, 1993; and Brent W094/10300), to identify other proteins which bind to or interact with the histamine H2-like GPCR polypeptide and modulate its activity.



The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a histamine H2-like GPCR polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein which interacts with the histamine H2-like GPCR polypeptide.

It may be desirable to immobilize either the histamine H2-like GPCR polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the histamine H2-like GPCR polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the histamine H2-like GPCR polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a histamine H2-like GPCR polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the

reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

5 In one embodiment, the histamine H2-like GPCR polypeptide is a fusion protein comprising a domain that allows the histamine H2-like GPCR polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed histamine H2-like GPCR  
10 polypeptide; the mixture is then incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from  
15 the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a histamine H2-like GPCR polypeptide (or polynucleotide) or a test compound can be  
20 immobilized utilizing conjugation of biotin and streptavidin. Biotinylated histamine H2-like GPCR polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively,  
25 antibodies which specifically bind to a histamine H2-like GPCR polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the histamine H2-like GPCR polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

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Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the histamine H2-like GPCR polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the histamine H2-like GPCR polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a histamine H2-like GPCR polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a histamine H2-like GPCR polypeptide or polynucleotide can be used in a cell-based assay system. A histamine H2-like GPCR polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a histamine H2-like GPCR polypeptide or polynucleotide is determined as described above.

15

#### Functional Assays

Test compounds can be tested for the ability to increase or decrease a biological effect of a histamine H2-like GPCR polypeptide. Such biological effects can be determined using the functional assays described in the specific examples, below. Functional assays can be carried out after contacting either a purified histamine H2-like GPCR polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound which decreases a functional activity of a histamine H2-like GPCR by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential agent for decreasing histamine H2-like GPCR activity. A test compound which increases histamine H2-like GPCR activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential agent for increasing histamine H2-like GPCR activity.

One such screening procedure involves the use of melanophores which are transfected to express a histamine H2-like GPCR polypeptide. Such a screening

technique is described in WO 92/01810 published Feb. 6, 1992. Thus, for example, such an assay may be employed for screening for a compound which inhibits activation of the receptor polypeptide by contacting the melanophore cells which comprise the receptor with both the receptor ligand (*e.g.*, histamine or a histamine analog) and a test compound to be screened. Inhibition of the signal generated by the ligand indicates that a test compound is a potential antagonist for the receptor, *i.e.*, inhibits activation of the receptor. The screen may be employed for identifying a test compound which activates the receptor by contacting such cells with compounds to be screened and determining whether each test compound generates a signal, *i.e.*, activates the receptor.

Other screening techniques include the use of cells which express a human histamine H2-like GPCR polypeptide (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation (*see, e.g., Science* 246, 181-296, 1989). For example, test compounds may be contacted with a cell which expresses a human histamine H2-like GPCR polypeptide and a second messenger response, *e.g.*, signal transduction or pH changes, can be measured to determine whether the test compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding a human histamine H2-like GPCR polypeptide into *Xenopus* oocytes to transiently express the receptor. The transfected oocytes can then be contacted with the receptor ligand and a test compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for test compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing a human histamine H2-like GPCR polypeptide in cells in which the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as described above by quantifying the degree of activation of the receptor from changes in the phospholipase activity.

Details of functional assays such as those described above are provided in the specific examples, below.

5     Histamine H2-like GPCR Gene Expression

In another embodiment, test compounds which increase or decrease histamine H2-like GPCR gene expression are identified. A histamine H2-like GPCR polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the histamine H2-like GPCR polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

20     The level of histamine H2-like GPCR mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide products of a histamine H2-like GPCR polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a histamine H2-like GPCR polypeptide.

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Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell which expresses a histamine H2-like GPCR polynucleotide can be used in a cell-based assay system. The histamine H2-like GPCR polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those  
5 described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

#### Pharmaceutical Compositions

10 The invention also provides pharmaceutical compositions which can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a histamine H2-like GPCR polypeptide, histamine H2-like GPCR polynucleotide, antibodies which specifically bind to a histamine H2-like GPCR polypeptide, or mimetics, agonists, antagonists, or  
15 inhibitors of a histamine H2-like GPCR polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in  
20 combination with other agents, drugs or hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be  
25 used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using  
30 pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be

formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

5 Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose,  
10 hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

15 Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity  
20 of active compound, *i.e.*, dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a  
25 filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

30 Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks'

solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection  
5 suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated  
10 solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a  
15 manner that is known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in  
20 aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

25 Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of  
30 administration.



Therapeutic Indications and Methods

GPCRs are ubiquitous in the mammalian host and are responsible for many biological functions, including many pathologies. Accordingly, it is desirable to find compounds and drugs which stimulate a GPCR on the one hand and which can inhibit the function of a GPCR on the other hand. For example, compounds which activate a GPCR may be employed for therapeutic purposes, such as the treatment of asthma, Parkinson's disease, acute heart failure, urinary retention, and osteoporosis. In particular, compounds which activate GPCRs are useful in treating various cardiovascular ailments such as caused by the lack of pulmonary blood flow or hypertension. In addition these compounds may also be used in treating various physiological disorders relating to abnormal control of fluid and electrolyte homeostasis and in diseases associated with abnormal angiotensin-induced aldosterone secretion.

In general, compounds which inhibit activation of a GPCR can be used for a variety of therapeutic purposes, for example, for the treatment of hypotension and/or hypertension, angina pectoris, myocardial infarction, ulcers, asthma, allergies, benign prostatic hypertrophy, and psychotic and neurological disorders including schizophrenia, manic excitement, depression, delirium, dementia or severe mental retardation, dyskinesias, such as Huntington's disease or Tourett's syndrome, among others. Compounds which inhibit GPCRs also are useful in reversing endogenous anorexia, in the control of bulimia, and in treating various cardiovascular ailments such as caused by excessive pulmonary blood flow or hypotension.

Antagonists which specifically bind Histamine H<sub>2</sub>-like GPCR may be administered to a subject's gastric mucosa in conjunction with a suitable pharmaceutical carrier. Control of histamine H<sub>2</sub>-like GPCR expression and activity may be used to treat duodenal ulcers and may be especially useful in combination with other therapeutic agents, such as the antacid and antibiotic regimes used to eliminate *Helicobacter pylori* and cure peptic ulcers. Reducing the amount of gastric acids secreted at meal-

time as well as between meals will provide the time and a noncorrosive environment in which cells of the gastric mucosa can heal. In some cases, combinations of therapeutic agents having different cellular mechanisms of action and potentially synergistic effects will allow the use of lower effective doses of each agent with lessening side effects.

In another embodiment, antagonists of histamine H<sub>2</sub>-like GPCR may be administered to the lungs of a subject in those cases in which the hyperactivity of histamine H<sub>2</sub>-like GPCR is triggering allergic or asthmatic responses. In both responses, the ability to modulate histamine H<sub>2</sub>-like GPCR activity may prevent the degranulation of excess numbers of monocytes, macrophages and other immune system cells that release the enzymes which produce unwarranted tissue destruction. Such therapy may also be warranted in the treatment of allergies, asthma, bronchitis, emphysema, aspergillosis, tuberculosis, and the like.

In another embodiment, antagonists of histamine H<sub>2</sub>-like GPCR may be administered to a subject for the modulation of muscle spasms and cramping associated with gastric distress including but not limited to gastritis, flu, colitis, Crohn's disease and the like.

The ubiquitous expression accross the nervous system (shown in Fig. 3 and 4.) stresses that the histamine H<sub>2</sub>-like GPCR can be regulated to treat various disorders of the nervous system. By far the highest level of expression is in fetal brain and adult brain. Detailed investigation of the expression of the receptor in various regions of adult brain and spinal cord reveals ubiquitous expression across the CNS, with cerebellum and cortex exhibiting the highest level of expression. Histamine H<sub>2</sub>-like GPCR inhibitors can therefore also be employed to treat disorders of the nervous system. Disorders of the nervous system which may be treated include brain injuries, cerebrovascular diseases and their consequences, Parkinson's disease, corticobasal degeneration, motor neuron disease, dementia, including ALS, multiple sclerosis, traumatic brain injury, stroke, post-stroke, post-traumatic brain injury, and

small-vessel cerebrovascular disease. Dementias, such as Alzheimer's disease, vascular dementia, dementia with Lewy bodies, frontotemporal dementia and Parkinsonism linked to chromosome 17, frontotemporal dementias, including Pick's disease, progressive nuclear palsy, corticobasal degeneration, Huntington's disease, 5 thalamic degeneration, Creutzfeld-Jakob dementia, HIV dementia, schizophrenia with dementia, and Korsakoff's psychosis also can be treated. Similarly, it may be possible to treat cognitive-related disorders, such as mild cognitive impairment, age-associated memory impairment, age-related cognitive decline, vascular cognitive impairment, attention deficit disorders, attention deficit hyperactivity disorders, and 10 memory disturbances in children with learning disabilities, by regulating the activity of P2Y-like receptors.

Pain that is associated with nervous system disorders also can be treated by regulating the activity of histamine H<sub>2</sub>-like GPCR. Pain which can be treated 15 includes that associated with central nervous system disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (*e.g.*, infarct, hemorrhage, vascular malformation). Non-central neuropathic pain includes that associated with post mastectomy pain, reflex 20 sympathetic dystrophy (RSD), trigeminal neuralgia, radioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (*e.g.*, diabetic neuropathy) vasculitic neuropathy (*e.g.* secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, and 25 post-herpetic neuralgia. Pain associated with cancer and cancer treatment also can be treated, as can headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania. By regulation of the histamine H<sub>2</sub>-like GPCR one can also treat visceral pain as 30 pancreatitis, intestinal cystitis, dysmenorrhea, irritable Bowel syndrome, Crohn's disease, biliary colic, ureteral colic, myocardial infarction and pain syndromes of the

pelvic cavity, e.g. vulvodynia, orchialgia, urethral syndrome and protatodynia. Histamine H2-like GPCR can also be used to treat acute pain for example postoperative pain and pain after trauma.

- 5 Antagonists may be administered to a subject to treat infections or inflammation of the urinary tract and bladder, and may administered to women with pelvic inflammatory disease with the intent of preventing infertility.

10 This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a histamine H2-like GPCR polypeptide binding molecule) can be used in an animal model to  
15 determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

20

A reagent which affects histamine H2-like GPCR activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce histamine H2-like GPCR activity. The reagent preferably binds to an expression product of a human histamine H2-like GPCR gene. If the expression product is a protein, the reagent is preferably an  
25 antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells which have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

30 In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about

30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5  $\mu\text{g}$  of DNA per 16 nmole of liposome delivered to about  $10^6$  cells, more preferably about 1.0  $\mu\text{g}$  of DNA per 16 nmole of liposome delivered to about  $10^6$  cells, and even more preferably about 2.0  $\mu\text{g}$  of DNA per 16 nmol of liposome delivered to about  $10^6$  cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a tumor cell, such as a tumor cell ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods which are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1  $\mu\text{g}$  to about 10  $\mu\text{g}$  of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5  $\mu\text{g}$  to about 5  $\mu\text{g}$  of polynucleotides are combined with about 8 nmol

liposomes, and even more preferably about 1.0  $\mu$ g of polynucleotides is combined with about 8 nmol liposomes.

5 In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al.* *Trends in Biotechnol.* 11, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* 263, 621-24 (1988); Wu *et al.*, *J. Biol. Chem.* 269, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci.* 10 *U.S.A.* 87, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem.* 266, 338-42 (1991).

#### Determination of a Therapeutically Effective Dose

15 The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which increases or decreases histamine H<sub>2</sub>-like GPCR activity relative to the histamine H<sub>2</sub>-like GPCR activity which occurs in the absence of the therapeutically effective dose.

20 For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

25 Therapeutic efficacy and toxicity, *e.g.*, ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it 30 can be expressed as the ratio, LD<sub>50</sub>/ED<sub>50</sub>.

Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED<sub>50</sub> with  
5 little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to  
10 provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be  
15 administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular  
20 dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

25 If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads,  
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synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

- 5 Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

### Diagnostic Methods

10

In another embodiment, antibodies which specifically bind histamine H2-like GPCR may be used for the diagnosis of conditions or diseases characterized by expression of histamine H2-like GPCR, or in assays to monitor patients being treated with histamine H2-like GPCR, agonists, antagonists or inhibitors. The antibodies useful  
15 for diagnostic purposes may be prepared in the same manner as those described above. Diagnostic assays for histamine H2-like GPCR include methods which utilize the antibody and a label to detect histamine H2-like GPCR in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a  
20 reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols including ELISA, RIA, and FACS for measuring histamine H2-like GPCR are known in the art and provide a basis for diagnosing altered or  
25 abnormal levels of histamine H2-like GPCR expression. Normal or standard values for histamine H2-like GPCR expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to histamine H2-like GPCR under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various  
30 methods, such as photometric methods. Quantities of histamine H2-like GPCR expressed in subject, control and disease, samples from biopsied tissues are

compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

5 In another embodiment of the invention, polynucleotides encoding histamine H2-like GPCR are used for diagnostic purposes. Polynucleotides which can be used include oligonucleotide sequences, as well as antisense RNA and DNA . Polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of histamine H2-like GPCR may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess  
10 expression of histamine H2-like GPCR, and to monitor regulation of histamine H2-like GPCR levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding histamine H2-like  
15 GPCR or closely related molecules, may be used to identify nucleic acid sequences which encode histamine H2-like GPCR. The specificity of the probe, whether it is made from a highly specific region, *e.g.*, 10 unique nucleotides in the 5' regulatory region, or a less specific region, *e.g.*, especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low)  
20 will determine whether the probe identifies only naturally occurring sequences encoding histamine H2-like GPCR, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the histamine H2-like GPCR  
25 encoding sequences. The hybridization probes may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NO:1 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring histamine H2-like GPCR.

30 Means for producing specific hybridization probes for DNAs encoding Histamine H2-like GPCR include the cloning of nucleic acid sequences encoding histamine H2-

like GPCR or histamine H2-like GPCR derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes *in vitro* by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides.

5 Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

10 Polynucleotide sequences encoding histamine H2-like GPCR may be used for the diagnosis of any condition or disease which is associated with the expression and activity of histamine H2-like GPCR. Examples of such conditions include, but are not limited to damage, disorders or diseases of the digestive, immune, respiratory, reproductive, urinary peripheral or central nervous systems. The level at which histamine H2-like GPCR is expressed or active in the endothelium of an artery or

15 vein may correlate with the immune system interactions in arteriosclerosis and present an opportunity to diagnose those events. Similarly endothelial/epithelial and immune cell interactions may be monitored in the lung, bladder, or gastric mucosa as they correlate with conditions or diseases affecting those structures.

20 Polynucleotide sequences encoding histamine H2-like GPCR may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dip stick, pin, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered histamine H2-like GPCR expression. Such qualitative or quantitative methods are well known in the art.

25 In a particular aspect, the nucleotide sequences encoding histamine H2-like GPCR may be useful in assays that detect activation or induction of various cancers, particularly those mentioned above. The nucleotide sequences encoding histamine H2-like GPCR may be labeled by standard methods, and added to a fluid or tissue

30 sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is

quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding histamine H2-like GPCR in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

To provide a basis for the diagnosis of disease associated with expression of histamine H2-like GPCR, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes histamine H2-like GPCR, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type

may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

5 Additional diagnostic uses for oligonucleotides designed from the sequences encoding histamine H2-like GPCR may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'.fwdarw.3') and another with antisense (3'.LAMBDA.5'),  
10 employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

15 Methods which may also be used to quantitate the expression of histamine H2-like GPCR include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby *et al.*, *J. Immunol. Method*, 159, 235-244, 1993; Duplaa *et al.* (1993) *Anal. Biochem.* 229-236, 1993). The speed of quantitation of multiple samples may be  
20 accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

In another embodiment of the invention, the nucleic acid sequences which encode  
25 histamine H2-like GPCR may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes,  
30 bacterial artificial chromosomes, bacterial P1 constructions or single chromosome

cDNA libraries as reviewed in Price, *Blood Rev.* 7, 127-134, 1993, and Trask, *Trends Genet.* 7, 149-154, 1991.

5 FISH (as described in Verma *et al.*, HUMAN CHROMOSOMES: A MANUAL OF BASIC  
TECHNIQUES, Pergamon Press, New York, N.Y., 1988) may be correlated with other  
physical chromosome mapping techniques and genetic map data. Examples of  
genetic map data can be found in the 1994 "Genome Issue" of *Science* (265:1981f).  
Correlation between the location of the gene encoding histamine H2-like GPCR on a  
physical chromosomal map and a specific disease, or predisposition to a specific  
10 disease, may help delimit the region of DNA associated with that genetic disease.  
The nucleotide sequences of the subject invention may be used to detect differences  
in gene sequences between normal, carrier, or affected individuals.

15 *In situ* hybridization of chromosomal preparations and physical mapping techniques  
such as linkage analysis using established chromosomal markers may be used for  
extending genetic maps. Often the placement of a gene on the chromosome of  
another mammalian species, such as mouse, may reveal associated markers even if  
the number or arm of a particular human chromosome is not known. New sequences  
can be assigned to chromosomal arms, or parts thereof, by physical mapping. This  
20 provides valuable information to investigators searching for disease genes using  
positional cloning or other gene discovery techniques. Once the disease or syndrome  
has been crudely localized by genetic linkage to a particular genomic region, for  
example, AT to 11q22-23 (Gatti *et al.*, *Nature* 336, 577-580, 1988), any sequences  
mapping to that area may represent associated or regulatory genes for further  
25 investigation. The nucleotide sequence of the subject invention may also be used to  
detect differences in the chromosomal location due to translocation, inversion, etc.  
among normal, carrier, or affected individuals.

30 All patents and patent applications cited in this disclosure are expressly incorporated  
herein by reference. The above disclosure generally describes the present invention.  
A more complete understanding can be obtained by reference to the following

specific examples which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

### EXAMPLE 1

5

#### *Detection of histamine H2-like GPCR activity*

The polynucleotide of SEQ ID NO: 1 is inserted into the expression vector pCEV4 and the expression vector pCEV4-histamine H2-like GPCR polypeptide obtained is  
10 transfected into human embryonic kidney 293 cells. The cells are scraped from a culture flask into 5 ml of Tris HCl, 5 mM EDTA, pH 7.5, and lysed by sonication. Cell lysates are centrifuged at 1000 rpm for 5 minutes at 4 °C. The supernatant is centrifuged at 30,000 x g for 20 minutes at 4 °C. The pellet is suspended in binding buffer containing 50 mM Tris HCl, 5 mM MgSO<sub>4</sub>, 1 mM EDTA, 100 mM NaCl, pH  
15 7.5, supplemented with 0.1 % BSA, 2 µg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 µg/ml phosphoramidon. Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10 % of an added radioligand, i.e. <sup>125</sup>I-labeled histamine H2, are added to 96-well polypropylene microtiter plates containing ligand, non-labeled peptides, and binding buffer to a final volume of 250  
20 µl.

In equilibrium saturation binding assays, membrane preparations are incubated in the presence of increasing concentrations (0.1 nM to 4 nM) of <sup>125</sup>I ligand.

25 Binding reaction mixtures are incubated for one hour at 30 °C. The reaction is stopped by filtration through GF/B filters treated with 0.5% polyethyleneimine, using a cell harvester. Radioactivity is measured by scintillation counting, and data are analyzed by a computerized non-linear regression program. Non-specific binding is defined as the amount of radioactivity remaining after incubation of membrane  
30 protein in the presence of 100 nM of unlabeled peptide. Protein concentration is measured by the Bradford method using Bio-Rad Reagent, with bovine serum

albumin as a standard. The histamine H2-like GPCR activity of the polypeptide comprising the amino acid sequence of SEQ ID NO: 2 is demonstrated.

## EXAMPLE 2

5

### *Radioligand binding assays*

Human embryonic kidney 293 cells transfected with a polynucleotide which expresses human histamine H2-like GPCR are scraped from a culture flask into 5 ml of Tris HCl, 5 mM EDTA, pH 7.5, and lysed by sonication. Cell lysates are centrifuged at 1000 rpm for 5 minutes at 4 °C. The supernatant is centrifuged at 30,000 x g for 20 minutes at 4 °C. The pellet is suspended in binding buffer containing 50 mM Tris HCl, 5 mM MgSO<sub>4</sub>, 1 mM EDTA, 100 mM NaCl, pH 7.5, supplemented with 0.1 % BSA, 2 µg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 µg/ml phosphoramidon. Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10 % of the added radioligand, i.e. histamine H2, are added to 96-well polypropylene microtiter plates containing <sup>125</sup>I-labeled ligand or test compound, non-labeled peptides, and binding buffer to a final volume of 250 µl.

20

In equilibrium saturation binding assays, membrane preparations are incubated in the presence of increasing concentrations (0.1 nM to 4 nM) of <sup>125</sup>I-labeled ligand or test compound (specific activity 2200 Ci/mmol). The binding affinities of different test compounds are determined in equilibrium competition binding assays, using 0.1 nM <sup>125</sup>I-peptide in the presence of twelve different concentrations of each test compound.

25

Binding reaction mixtures are incubated for one hour at 30 °C. The reaction is stopped by filtration through GF/B filters treated with 0.5% polyethyleneimine, using a cell harvester. Radioactivity is measured by scintillation counting, and data are analyzed by a computerized non-linear regression program.

30



Non-specific binding is defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of 100 nM of unlabeled peptide. Protein concentration is measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard. A test compound which increases the radioactivity of membrane protein by at least 15% relative to radioactivity of membrane protein which was not incubated with a test compound is identified as a compound which binds to a human histamine H2-like GPCR polypeptide.

### 10 EXAMPLE 3

#### *Effect of a test compound on human histamine H2-like GPCR -mediated cyclic AMP formation*

15 Receptor-mediated inhibition of cAMP formation can be assayed in host cells which express human histamine H2-like GPCR. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5 mM theophylline, 2 µg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 µg/ml phosphoramidon for 20 minutes at 37 °C in 5% CO<sub>2</sub>. A test compound is added and  
20 incubated for an additional 10 minutes at 37 °C. The medium is aspirated, and the reaction is stopped by the addition of 100 mM HCl. The plates are stored at 4 °C for 15 minutes. cAMP content in the stopping solution is measured by radioimmunoassay.

25 Radioactivity is quantified using a gamma counter equipped with data reduction software. A test compound which decreases radioactivity of the contents of a well relative to radioactivity of the contents of a well in the absence of the test compound is identified as a potential inhibitor of cAMP formation. A test compound which increases radioactivity of the contents of a well relative to radioactivity of the  
30 contents of a well in the absence of the test compound is identified as a potential enhancer of cAMP formation.

#### **EXAMPLE 4**

##### *Effect of a test compound on the mobilization of intracellular calcium*

5

Intracellular free calcium concentration can be measured by microspectrofluorometry using the fluorescent indicator dye Fura-2/AM (Bush *et al.*, *J. Neurochem.* 57, 562-74, 1991). Stably transfected cells are seeded onto a 35 mm culture dish containing a glass coverslip insert. Cells are washed with HBS, incubated with a test compound, and loaded with 100  $\mu$ l of Fura-2/AM (10  $\mu$ M) for 20-40 minutes. After washing with HBS to remove the Fura-2/AM solution, cells are equilibrated in HBS for 10-20 minutes. Cells are then visualized under the 40X objective of a Leitz Fluovert FS microscope.

15 Fluorescence emission is determined at 510 nm, with excitation wavelengths alternating between 340 nm and 380 nm. Raw fluorescence data are converted to calcium concentrations using standard calcium concentration curves and software analysis techniques. A test compound which increases the fluorescence by at least 15% relative to fluorescence in the absence of a test compound is identified as a  
20 compound which mobilizes intracellular calcium.

#### **EXAMPLE 5**

##### *Effect of a test compound on phosphoinositide metabolism*

25

Cells which stably express human histamine H2-like GPCR cDNA are plated in 96-well plates and grown to confluence. The day before the assay, the growth medium is changed to 100  $\mu$ l of medium containing 1% serum and 0.5  $\mu$ Ci  $^3$ H-myo-inositol. The plates are incubated overnight in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> at 37 °C).  
30 Immediately before the assay, the medium is removed and replaced by 200  $\mu$ l of PBS containing 10 mM LiCl, and the cells are equilibrated with the new medium for 20

minutes. During this interval, cells also are equilibrated with antagonist, added as a 10 µl aliquot of a 20-fold concentrated solution in PBS.

The <sup>3</sup>H-inositol phosphate accumulation from inositol phospholipid metabolism is started by adding 10 µl of a solution containing a test compound. To the first well 10 µl are added to measure basal accumulation. Eleven different concentrations of test compound are assayed in the following 11 wells of each plate row. All assays are performed in duplicate by repeating the same additions in two consecutive plate rows.

The plates are incubated in a CO<sub>2</sub> incubator for one hour. The reaction is terminated by adding 15 µl of 50% v/v trichloroacetic acid (TCA), followed by a 40 minute incubation at 4 °C. After neutralizing TCA with 40 µl of 1 M Tris, the content of the wells is transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates are prepared by adding 200 µl of Dowex AG1-X8 suspension (50% v/v, water:resin) to each well. The filter plates are placed on a vacuum manifold to wash or elute the resin bed. Each well is washed 2 times with 200 µl of water, followed by 2 x 200 µl of 5 mM sodium tetraborate/60 mM ammonium formate.

The <sup>3</sup>H-IPs are eluted into empty 96-well plates with 200 µl of 1.2 M ammonium formate/0.1 formic acid. The content of the wells is added to 3 ml of scintillation cocktail, and radioactivity is determined by liquid scintillation counting.

## **EXAMPLE 6**

### *Receptor Binding Methods*

Standard Binding Assays. Binding assays are carried out in a binding buffer containing 50 mM HEPES, pH 7.4, 0.5% BSA, and 5 mM MgCl<sub>2</sub>. The standard assay for radioligand binding to membrane fragments comprising histamine H<sub>2</sub>-like

GPCR polypeptides is carried out as follows in 96 well microtiter plates (e.g., Dynatech Immulon II Removawell plates). Radioligand is diluted in binding buffer+ PMSF/Baci to the desired cpm per 50  $\mu$ l, then 50  $\mu$ l aliquots are added to the wells. For non-specific binding samples, 5  $\mu$ l of 40  $\mu$ M cold ligand also is added per well.

5 Binding is initiated by adding 150  $\mu$ l per well of membrane diluted to the desired concentration (10-30  $\mu$ g membrane protein/well) in binding buffer+ PMSF/Baci. Plates are then covered with Linbro mylar plate sealers (Flow Labs) and placed on a Dynatech Microshaker II. Binding is allowed to proceed at room temperature for 1-2 hours and is stopped by centrifuging the plate for 15 minutes at 2,000 x g. The

10 supernatants are decanted, and the membrane pellets are washed once by addition of 200  $\mu$ l of ice cold binding buffer, brief shaking, and recentrifugation. The individual wells are placed in 12 x 75 mm tubes and counted in an LKB Gammamaster counter (78% efficiency). Specific binding by this method is identical to that measured when free ligand is removed by rapid (3-5 seconds) filtration and washing on

15 polyethyleneimine-coated glass fiber filters.

Three variations of the standard binding assay are also used.

- 20 1. Competitive radioligand binding assays with a oncentration range of cold ligand vs. <sup>125</sup> I-labeled ligand are carried out as described above with one modification. All dilutions of ligands being assayed are made in 40X PMSF/Baci to a concentration 40X the final concentration in the assay. Samples of peptide (5  $\mu$ l each) are then added per microtiter well. Membranes and radioligand are diluted in binding buffer without protease inhibitors.
- 25 Radioligand is added and mixed with cold ligand, and then binding is initiated by addition of membranes.
2. Chemical cross-linking of radioligand with receptor is done after a binding step identical to the standard assay. However, the wash step is done with
- 30 binding buffer minus BSA to reduce the possibility of non-specific cross-

linking of radioligand with BSA. The cross-linking step is carried out as described below.

3. Larger scale binding assays to obtain membrane pellets for studies on solubilization of receptor:ligand complex and for receptor purification are also carried out. These are identical to the standard assays except that (a) binding is carried out in polypropylene tubes in volumes from 1-250 ml, (b) concentration of membrane protein is always 0.5 mg/ml, and (c) for receptor purification, BSA concentration in the binding buffer is reduced to 0.25%, and the wash step is done with binding buffer without BSA, which reduces BSA contamination of the purified receptor.

#### EXAMPLE 7

##### 15 *Chemical Cross-Linking of Radioligand to Receptor*

After a radioligand binding step as described above, membrane pellets are resuspended in 200 µl per microtiter plate well of ice-cold binding buffer without BSA. Then 5 µl per well of 4 mM N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS, Pierce) in DMSO is added and mixed. The samples are held on ice and UV-irradiated for 10 minutes with a Mineralight R-52G lamp (UVP Inc., San Gabriel, Calif.) at a distance of 5-10 cm. Then the samples are transferred to Eppendorf microfuge tubes, the membranes pelleted by centrifugation, supernatants removed, and membranes solubilized in Laemmli SDS sample buffer for polyacrylamide gel electrophoresis (PAGE). PAGE is carried out as described below. Radiolabeled proteins are visualized by autoradiography of the dried gels with Kodak XAR film and Dupont image intensifier screens.

### EXAMPLE 8

#### *Membrane Solubilization*

Membrane solubilization is carried out in buffer containing 25 mM Tris , pH 8, 10% glycerol (w/v) and 0.2 mM CaCl<sub>2</sub> (solubilization buffer). The highly soluble detergents including Triton X-100, deoxycholate, deoxycholate:lysolecithin, CHAPS, and zwittergent are made up in solubilization buffer at 10% concentrations and stored as frozen aliquots. Lysolecithin is made up fresh because of insolubility upon freeze-thawing and digitonin is made fresh at lower concentrations due to its more limited solubility.

To solubilize membranes, washed pellets after the binding step are resuspended free of visible particles by pipetting and vortexing in solubilization buffer at 100,000 x g for 30 minutes. The supernatants are removed and held on ice and the pellets are discarded.

### EXAMPLE 9

#### *Assay of Solubilized Receptors*

After binding of <sup>125</sup>I ligands and solubilization of the membranes with detergent, the intact R:L complex can be assayed by four different methods. All are carried out on ice or in a cold room at 4-10 °C.).

1. Column chromatography (Knuhtsen *et al.*, *Biochem. J.* 254, 641-647, 1988). Sephadex G-50 columns (8 x 250 mm) are equilibrated with solubilization buffer containing detergent at the concentration used to solubilize membranes and 1 mg/ml bovine serum albumin. Samples of solubilized membranes (0.2-0.5 ml) are applied to the columns and eluted at a flow rate of about 0.7 ml/minute. Samples (0.18 ml) are collected. Radioactivity is determined in a gamma counter. Void volumes of the columns are determined by the elution

volume of blue dextran. Radioactivity eluting in the void volume is considered bound to protein. Radioactivity eluting later, at the same volume as free  $^{125}\text{I}$  ligands, is considered non-bound.

- 5        2.    Polyethyleneglycol precipitation (Cuatrecasas, *Proc. Natl. Acad. Sci. USA* 69, 318-322, 1972). For a 100  $\mu\text{l}$  sample of solubilized membranes in a 12 x 75 mm polypropylene tube, 0.5 ml of 1% (w/v) bovine gamma globulin (Sigma) in 0.1 M sodium phosphate buffer is added, followed by 0.5 ml of 25% (w/v) polyethyleneglycol (Sigma) and mixing. The mixture is held on ice for 15  
10        minutes. Then 3 ml of 0.1 M sodium phosphate, pH 7.4, is added per sample. The samples are rapidly (1-3 seconds) filtered over Whatman GF/B glass fiber filters and washed with 4 ml of the phosphate buffer. PEG-precipitated receptor :  $^{125}\text{I}$ -ligand complex is determined by gamma counting of the filters.
- 15        3.    GFB/PEI filter binding (Bruns *et al.*, *Analytical Biochem.* 132, 74-81, 1983). Whatman GF/B glass fiber filters are soaked in 0.3% polyethyleneimine (PEI, Sigma) for 3 hours. Samples of solubilized membranes (25-100  $\mu\text{l}$ ) are replaced in 12 x 75 mm polypropylene tubes. Then 4 ml of solubilization  
20        buffer without detergent is added per sample and the samples are immediately filtered through the GFB/PEI filters (1-3 seconds) and washed with 4 ml of solubilization buffer. CPM of receptor :  $^{125}\text{I}$ -ligand complex adsorbed to filters are determined by gamma counting.
- 25        4.    Charcoal/Dextran (Paul and Said, *Peptides* 7[Suppl. 1], 147-149, 1986). Dextran T70 (0.5 g, Pharmacia) is dissolved in 1 liter of water, then 5 g of activated charcoal (Norit A, alkaline; Fisher Scientific) is added. The suspension is stirred for 10 minutes at room temperature and then stored at 4°C. until use. To measure R:L complex, 4 parts by volume of  
30        charcoal/dextran suspension are added to 1 part by volume of solubilized membrane. The samples are mixed and held on ice for 2 minutes and then centrifuged for 2 minutes at 11,000 x g in a Beckman microfuge. Free

radioligand is adsorbed charcoal/dextran and is discarded with the pellet.  
Receptor :  $^{125}$ I-ligand complexes remain in the supernatant and are determined by gamma counting.

5     **EXAMPLE 10**

*Receptor Purification*

Binding of biotinyl-receptor to GH<sub>4</sub> C1 membranes is carried out as described above.  
10     Incubations are for 1 hour at room temperature. In the standard purification protocol, the binding incubations contain 10 nM Bio-S29.  $^{125}$ I ligand is added as a tracer at levels of 5,000-100,000 cpm per mg of membrane protein. Control incubations contain 10  $\mu$ M cold ligand to saturate the receptor with non-biotinylated ligand.

15     Solubilization of receptor:ligand complex also is carried out as described above, with 0.15% deoxycholate:lysolecithin in solubilization buffer containing 0.2 mM MgCl<sub>2</sub>, to obtain 100,000 x g supernatants containing solubilized R:L complex.

20     Immobilized streptavidin (streptavidin cross-linked to 6% beaded agarose, Pierce Chemical Co.; "SA-agarose") is washed in solubilization buffer and added to the solubilized membranes as 1/30 of the final volume. This mixture is incubated with constant stirring by end-over-end rotation for 4-5 hours at 4-10 °C. Then the mixture is applied to a column and the non-bound material is washed through. Binding of radioligand to SA-agarose is determined by comparing cpm in the 100,000 x g  
25     supernatant with that in the column effluent after adsorption to SA-agarose. Finally, the column is washed with 12-15 column volumes of solubilization buffer+0.15% deoxycholate:lysolecithin +1/500 (vol/vol) 100 x 4pase.

30     The streptavidin column is eluted with solubilization buffer+0.1 mM EDTA+0.1 mM EGTA+0.1 mM GTP-gamma-S (Sigma)+0.15% (wt/vol) deoxycholate:lysolecithin +1/1000 (vol/vol) 100.times.4pase. First, one column volume of elution buffer is



passed through the column and flow is stopped for 20-30 minutes. Then 3-4 more column volumes of elution buffer are passed through. All the eluates are pooled.

5 Eluates from the streptavidin column are incubated overnight (12-15 hours) with immobilized wheat germ agglutinin (WGA agarose, Vector Labs) to adsorb the receptor via interaction of covalently bound carbohydrate with the WGA lectin. The ratio (vol/vol) of WGA-agarose to streptavidin column eluate is generally 1:400. A range from 1:1000 to 1:200 also can be used. After the binding step, the resin is pelleted by centrifugation, the supernatant is removed and saved, and the resin is  
10 washed 3 times (about 2 minutes each) in buffer containing 50 mM HEPES, pH 8, 5 mM  $MgCl_2$  and 0.15% deoxycholate:lysolecithin. To elute the WGA-bound receptor, the resin is extracted three times by repeated mixing (vortex mixer on low speed) over a 15-30 minute period on ice, with 3 resin columns each time, of 10 mM N-N'-N''-triacetylchitotriose in the same HEPES buffer used to wash the resin. After  
15 each elution step, the resin is centrifuged down and the supernatant is carefully removed, free of WGA-agarose pellets. The three, pooled eluates contain the final, purified receptor. The material non-bound to WGA contain G protein subunits specifically eluted from the streptavidin column, as well as non-specific contaminants. All these fractions are stored frozen at -90 °C.

20

### EXAMPLE 11

#### Quantitative analysis of the relative receptor expression in human tissues

25 Quantitative expression profiling was performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi et al., 1992 and Higuchi et al., 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

30

If the amplification is performed in the presence of an internally quenched fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland et al.). Since the fluorescence emission will increase in direct proportion to the amount of the specific amplified product, the exponential growth phase of PCR product can be detected and used to determine the initial template concentration (Heid et al., 1996, and Gibson et al., 1996).

The amplification of an endogenous control can be performed to standardise the amount of sample RNA added to a reaction. In this kind of experiments the control of choice is the 18S ribosomal RNA. Since reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently quantified in the same tube if probes labelled with different dyes are used.

All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700 Sequence detector System (PE Applied Biosystems, Foster City, CA).

#### References

- Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10:413-417.
- Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. . *BioTechnology* 11:1026-1030.
- Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc.Natl.Acad.Sci.* 88:7276-7280.

- Heid, C., Stevens, J., Livak, K. And Williams, P.M. 1996. Real time quantitative PCR. *Genome Res.* 6:986-994.
- Gibson, U.E., Heid, C.A. and Williams, P.M. 1996. A novel method for real time quantitative RT-PCR. *Genome Res.* 6: 995-1001.

5

#### cDNA preparation

The total RNAs used for expression quantification are listed in Table 1 along with their purchasers.

10

Fifty µg of each RNA were treated with DNase I for 1 hour at 37°C in the following reaction mix:

DNase I, RNase-free (Roche Diagnostics, Germany)      0.2 U/µL

15

Rnase inhibitor (PE Applied Biosystems, CA)      0.4 U/µL

Tris-HCl pH 7.9      10mM

20      MgCl<sub>2</sub>      10mM

NaCl      50mM

DTT      1mM

25      After incubation, RNA was extracted once with 1 volume of phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of NaAcetate 3M pH5.2 and 2 volume ethanol.

30      After spectrophotometric quantification, each sample has been reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) accordingly to purchaser protocol. RNA final concentration in the reaction mix was 200ng/µL. Reverse transcription was made with 2.5µM of random hexamers.

TaqMan quantitative analysis

5 Specific primers and probe were designed accordingly to PE Applied Biosystems recommendations and are listed below:

forward primer: 5'-AACTGGGCAGGTGGAGAGTGT-3'

reverse primer: 5'-TGCCGGTTGAGACATCCATAG-3'

10 probe: 5'-(FAM) ATTGGCTACTTTTGCTTCACTTCCAACCCTTTC (TAMRA)-  
3'

where FAM = 6-carboxy-fluorescein

and TAMRA = 6-carboxy-tetramethyl-rhodamine.

15 The expected length of the PCR product was 87bp.

Quantification experiments were performed on 10 ng of reverse transcribed RNA from each sample. Each determination was done in triplicate.

20 Total cDNA content was normalized with the simultaneous quantification (multiplex PCR) of the 18S ribosomal RNA by use of the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

Assay reaction mix was as follows:

	final	
25	TaqMan Universal PCR Master Mix (2x)	1x
	PDAR control – 18S RNA (20x	1x
	Forward primer 900nM	
	Reverse primer 900nM	
	Probe 200nM	
30	cDNA 10ng	
	Water to 25µL	

PCR conditions were:

1 time the following steps:

pre PCR        2' at 50° C

5                    10' at 95°C

40 times the following steps:

denaturation   15'' at 95°C

10                annealing/extension   1' at 60°C

The experiment was performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR were processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target quantity.

The background value subtracted was the same in independent experiments, to allow inter-experiment comparisons of the expression levels.

20

The relative expression data are shown in the graph and expressed as "relative expression units", i.e. as fold over the 18S endogenous control expression multiplied to a numerical factor ( $10^7$ ) in order to obtain values higher than 1.

25        Raw data and analysis of relative expression are also shown in the table below the graph.

RNA	Purch. & catalogue #	Lot #
h. Foetal Brain	Clontech (CA) 640191	6120259
h. Brain	OriGene (MD) HT1001	9802R
h. Muscle	OriGene (MD) HT1008	98229R
h. Muscle	from autopsy	-
h. Heart	OriGene (MD) HT1002	9901
h. Lung	OriGene (MD) HT1009	98605W
h. Kidney	OriGene (MD) HT1003	98514R
h. Kidney	from autopsy	-
h. Liver	OriGene (MD) HT1005	9205
h. Liver	from autopsy	-
h. Thymus	Clontech (CA) 640281	9080955
h. Testis	OriGene (MD) HT1011	9903
h. Colon	from autopsy	-
h. Trachea	Clontech 640911	9110410
h. Pancreas	Clontech 640311	8120795
h. Gastric mucose	from autopsy	-
h. Foetal Liver	Clontech (CA) 640181	9031140
h. Bladder	Invitrogen (CA) D602001	A212095
h. Adrenal gland	Clontech (CA) 640161	9080594
h. Spleen	Origene (HT)1004	980413G
h. Prostate	Clontech (CA) 640381	9031142
h. Prostate	from autopsy	-
h. hypertrophic prostate	from autopsy	-
h. monocytes	from blood subset	-
h. macrophages	from blood subset	-
SMC	Cultured cell line	-
hUVEC	Cultured cell line	-
h.heart	From autopsy	-

RNA	Purch. & catalogue #	Lot #
h. brain	from autopsy	-
h. Cortex	from autopsy	-
h. Alzheimer cortex	from autopsy	-
h. Choroid plexus	from autopsy	-
h. Hippocampus	from autopsy	-
h. Hypothalamus	from autopsy	-
h. Amygdala	from autopsy	-
h. Thalamus	from autopsy	-
h. Cerebellum	from autopsy	-
h. Cerebellum	Clontech (CA) 640351	8120791
h. Spinal Cord	Clontech (CA) K40031	40718

**EXAMPLE 12***Identification of test compounds that bind to histamine H<sub>2</sub>-like GPCR polypeptides*

5

Purified histamine H<sub>2</sub>-like GPCR polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well micro-titer plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. histamine H<sub>2</sub>-like GPCR polypeptides comprise an amino acid sequence shown in SEQ ID NO:2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

10

15

The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a histamine H<sub>2</sub>-like GPCR polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound which increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a histamine H<sub>2</sub>-like GPCR polypeptide.

**EXAMPLE 13**

*Identification of a test compound which decreases histamine H2-like GPCR gene expression*

A test compound is administered to a culture of human gastric cells and incubated at 37 °C for 10 to 45 minutes. A culture of the same type of cells incubated for the same time without the test compound provides a negative control.

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem.* 18, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a <sup>32</sup>P-labeled histamine H2-like GPCR-specific probe at 65 °C in Express- hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO:1. A test compound which decreases the histamine H2-like GPCR-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of histamine H2-like GPCR gene expression.

**EXAMPLE 14**

*Treatment of peptic ulcer with a reagent which specifically binds to a histamine H2-like GPCR gene product*

Synthesis of antisense histamine H2-like GPCR oligonucleotides comprising at least 11 contiguous nucleotides selected from the complement of SEQ ID NO:1 is performed on a Pharmacia Gene Assembler series synthesizer using the phosphoramidite procedure (Uhlmann *et al.*, *Chem. Rev.* 90, 534-83, 1990). Following assembly and deprotection, oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate-buffered saline (PBS) at the desired concentration. Purity of these oligonucleotides is tested by capillary gel electro-



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phoreses and ion exchange HPLC. Endotoxin levels in the oligonucleotide preparation are determined using the Limulus Amebocyte Assay (Bang, *Biol. Bull. (Woods Hole, Mass.)* 105, 361-362, 1953).

- 5 The antisense oligonucleotides are administered to a patient with a peptide ulcer. The severity of the patient's peptide ulcer is lessened.

**CLAIMS**

1. An isolated polynucleotide encoding a histamine H2-like GPCR polypeptide  
5 and being selected from the group consisting of:
- a) a polynucleotide encoding a histamine H2-like GPCR polypeptide  
comprising an amino acid sequence selected from the group consisting  
of:  
10 amino acid sequences which are at least about 50% identical to  
the amino acid sequence shown in SEQ ID NO: 2; and  
the amino acid sequence shown in SEQ ID NO: 2.
- b) a polynucleotide comprising the sequence of SEQ ID NO: 1;
- c) a polynucleotide which hybridizes under stringent conditions to a  
15 polynucleotide specified in (a) and (b);
- d) a polynucleotide the sequence of which deviates from the  
polynucleotide sequences specified in (a) to (c) due to the  
degeneration of the genetic code; and
- e) a polynucleotide which represents a fragment, derivative or allelic  
20 variation of a polynucleotide sequence specified in (a) to (d).
2. An expression vector containing any polynucleotide of claim 1.
3. A host cell containing the expression vector of claim 2.
- 25 4. A substantially purified histamine H2-like GPCR polypeptide encoded by a  
polynucleotide of claim 1.
5. A method for producing a histamine H2-like GPCR polypeptide, wherein the  
30 method comprises the following steps:

- a) culturing the host cell of claim 3 under conditions suitable for the expression of the histamine H2-like GPCR polypeptide; and
- b) recovering the histamine H2-like GPCR polypeptide from the host cell culture.

5

6. A method for detection of a polynucleotide encoding a histamine H2-like GPCR polypeptide in a biological sample comprising the following steps:

- a) hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and
- b) detecting said hybridization complex.

10

7. The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.

15

8. A method for the detection of a polynucleotide of claim 1 or a histamine H2-like GPCR polypeptide of claim 5 comprising the steps of contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the histamine H2-like GPCR polypeptide.

20

9. A diagnostic kit for conducting the method of any one of claims 6 to 8.

10. A method of screening for agents which decrease the activity of a histamine H2-like GPCR, comprising the steps of:

25

contacting a test compound with any histamine H2-like GPCR polypeptide encoded by any polynucleotide of claim 1;

30

detecting binding of the test compound of the histamine H2-like GPCR polypeptide, wherein a test compound which binds to the polypeptide is

identified as a potential therapeutic agent for decreasing the activity of a histamine H2-like GPCR.

- 5 11. A method of screening for agents which regulate the activity of a histamine H2-like GPCR, comprising the steps of:  
contacting a test compound with a histamine H2-like GPCR polypeptide encoded by any polynucleotide of claim 1; and

10 detecting a histamine H2-like GPCR activity of the polypeptide, wherein a test compound which increases the histamine H2-like GPCR activity is identified as a potential therapeutic agent for increasing the activity of the histamine H2-like GPCR, and wherein a test compound which decreases the histamine H2-like GPCR activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the histamine H2-like  
15 GPCR.

12. A method of screening for agents which decrease the activity of a histamine H2-like GPCR, comprising the steps of:  
20 contacting a test compound with any polynucleotide of claim 1 and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of histamine H2-like GPCR.

- 25 13. A method of reducing the activity of histamine H2-like GPCR, comprising the steps of:

contacting a cell with a reagent which specifically binds to any polynucleotide of claim 1 or any histamine H2-like GPCR polypeptide of  
30 claim 4, whereby the activity of histamine H2-like GPCR is reduced.

14. A reagent that modulates the activity of a histamine H2-like GPCR polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the claims 10 to 12.
- 5 15. A pharmaceutical composition, comprising:
- the expression vector of claim 2 or the reagent of claim 14 and a pharmaceutically acceptable carrier.
- 10 16. Use of the pharmaceutical composition of claim 15 for modulating the activity of a histamine H2-like GPCR in a disease.
17. Use of claim 16 wherein the disease is a disease of the digestive, immune, respiratory, reproductive, urinary, peripheral or central nervous system.
- 15 18. Use of claim 17 wherein the disease is a primary or secondary disorder after brain injury, a disorder of mood, an anxiety disorder, a disorder of thought and volition, a disorder of sleep and wakefulness, a disease of the motor unit, a neurogenic and myopathic disorder, a neurodegenerative disorder,
- 20 Alzheimer's disease, Parkinson's disease, or a disorder leading to peripheral and chronic pain

- 1/4 -

Fig. 1

ACGAAACCAGGAAGCCTGGGCCTGGGCTCGCCATCCCAGGGTCGCTGGACTAGGATGGGGGATGGGCCTGTGACAGG  
AGGTACCCTGGGTGCCCTCTTTCGGCCCCATGGAGTCCTCACCCATCCCCAGTCATCAGGGAACTCTTCCACTTTGG  
GGAGGGTCCCTCAAACCCAGGTCCCTCTACTGCCAGTGGGGTCCCGGAGGTGGGGCTACGGGATGTTGCTTCGGAAT  
CTGTGGCCCTCTTCTTCATGCTCCTGCTGGACTTGACTGCTGTGGCTGGCAATGCCGCTGTGATGGCCGTGATCGCCAA  
GACGCCTGCCCTCCGAAAATTGTCTTCGTCTTCCACCTCTGCCTGGTGGACCTGCTGGCTGCCCTGACCCTCATGCCC  
CTGGCCATGCTCTCCAGCTCTGCCCTCTTTGACCACGCCCTCTTTGGGGAGGTGGCCTGCCGCCTCTACTTGTTTCTGAG  
CGTGTGCTTTGTGAGCCTGGCCATCCTCTCGGTGTCAGCCATCAATGTGGAGCGCTACTATTACGTAGTCCACCCCATG  
CGCTACGAGGTGCCATGACGCTGGGGCTGGTGGCTCTGTGCTGGTGGGTGTGTGGGTGAAGGCCTTGGCCATGGCT  
TCTGTGCCAGTGTGGGAAGGGTCTCCTGGGAGGAAGGAGCTCCCAGTGTCCCCCAGGCTGTTCACTCCAGTGGAGC  
CACAGTGCCTACTGCCAGCTTTTGTGGTGGTCTTTGCTGTCTTTACTTTCTGTTGCCCTGCTCCTCATACTTGTTGTC  
TACTGCAGCATGTTCCGAGTGGCCCGCGTGGCTGCCATGCAGCACGGGCCGCTGCCACGTGGATGGAGACACCCCGG  
CAACGCTCCGAATCTCTCAGCAGCCGCTCCACGATGGTCACCAGCTCGGGGGCCCCCAGACCACCCACACCGGACG  
TTTGGGGGAGGGAAAGCAGCAGTGGTTCTCCTGGCTGTGGGGGACAGTTCCTGCTCTGTTGGTTGCCCTACTTCTCTTT  
CCACCTCTATGTTGCCCTGAGTGCTCAGCCATTTCAACTGGGCAGGTGGAGAGTGTGGTCACCTGGATTGGCTACTTTT  
GCTTCACTTCCAACCTTTCTTCTATGGATGTCTCAACCGGCAGATCCGGGGGAGCTCAGCAAGCAGTTTGTCTGCTTC  
TTCAAGCCAGCTCCAGAGGAGGAGCTGAGGCTGCCTAGCCGGGAGGGCTCCATTGAGGAGAACTTCTGTCAGTTCCTT  
CAGGGGACTGGCTGTCTTCTGAGTCTGGGTTTCCCGACCCCTACCCAGCCCCAAGCAGGAGCCACCTGCTGTTGACT  
TTCGAATCCCAGGCCAGATAGCTGAGGAGACCTCTGAGTTCCTGGAGCAGCAACTCACCAGCGACATCATCATGTCAG  
ACAGCTACCTCCGTCTGCCGCCTCACCCCGGCTGGAGTCATGATGGGCCGCTGGACACTCGGAGGGATATGGG  
GCTGGGGCCAGTTATGATTGCAAGGACCACCTTGTGGGATCACCTTTTCCCAGCTGGCTAGGGCTGAGGCTGGGGTCTC  
TGCACACA

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Fig. 2

MESSPIQSSGNSSTLGRVPQTPGPSTASGVPEVGLRDVASESVALFFMLLLDLTAVAGNAAVMAVIAKTPAL  
RKFFVVFHLCCLVDLLAALTLMPLAMLSSSALFDHALFGEVACRLYLFLSVCFVSLAILSVSAINVERYYYVHP  
MRYEVRMTLGLVASVLVGWVKALAMASVPVLGRVSWEEGAPSVPPGCSLQWSHSAYCQLFVVVFAVLYF  
LLPLLLLVVYCSMFRVARVAAMQHGPLPTWMETPRQRSESLSSRSTMVTSSGAPQTTPHRTFGGGKAAVLL  
AVGGQFLLCWLPYFSFHLVVALSAQPISTGQVESVVTWIGYFCFTSNPFFYGCLNRQIRGELSKQFVCFFKPAP  
EEELRLPSREGSIEENFLQFLQGTGCPSESWVSRPLSPKQEPPAVDFRIPGQIAEETSEFLEQQLTSDIIMSDSYL  
RPAASPRLES

- 3/4 -

		avg. Ct	avg. Ct 18S	delta	raw fold	rel.expr.	Dev.Sta.	SD+	SD-
H105	Brain	29,0	12,3	16,7	9,5E-06	95,5	0,22	13,4	15,6
H107	Cortex	29,1	12,4	16,7	9,2E-06	91,8	0,20	11,9	13,7
H104	Cortex Alzheimer	30,1	12,5	17,7	4,8E-06	48,1	0,24	7,4	8,7
H111	Choroid plexus	32,4	13,6	18,8	2,3E-06	22,5	0,19	2,8	3,1
H110	Hippocampus	30,8	12,7	18,2	3,4E-06	33,8	0,31	6,5	8,1
H106	Hypothalamus	30,1	13,0	17,1	7,3E-06	72,7	0,23	10,6	12,4
H108	Amygdala	31,1	13,8	17,4	5,9E-06	59,3	0,15	6,0	6,7
H112	Thalamus	29,9	12,7	17,3	6,2E-06	62,3	0,26	10,3	12,4
H101	Cerebellum (pool)	28,5	12,1	16,3	1,2E-05	120,6	0,28	21,0	25,4
H109	Cerebellum	29,9	12,9	17,0	7,9E-06	78,6	0,20	10,0	11,4
H113	Spinal cord (pool)	32,4	13,3	19,2	1,7E-06	17,0	0,35	3,7	4,7

Fig. 3



Histamine H2-like GPCR - Human CNS Panel (endog. ref. 18S)

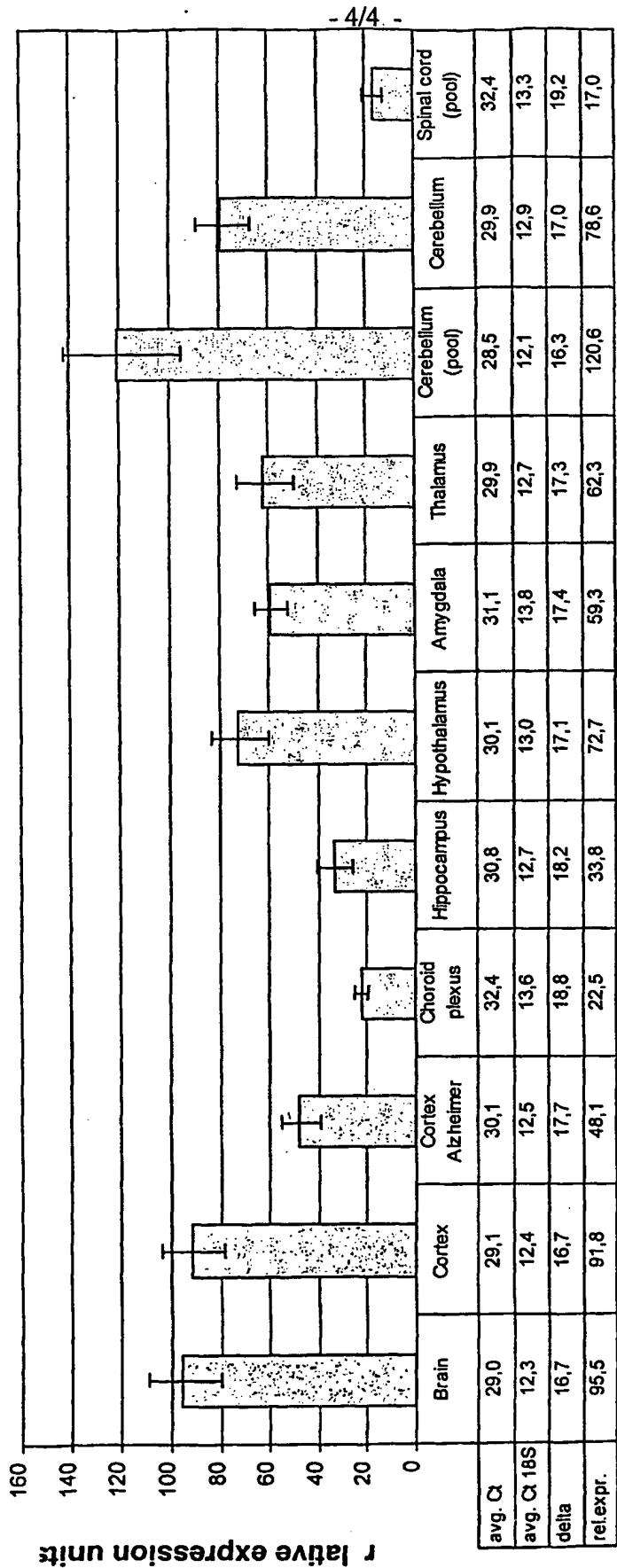


Fig. 4:

## SEQUENZPROTOKOLL

&lt;110&gt; Bayer AG

<120> REGULATION OF HUMAN HISTAMINE H2-LIKE G PROTEIN-COUPLED  
RECEPTOR

&lt;130&gt; Lio012 Foreign Countries

&lt;140&gt;

&lt;141&gt;

&lt;150&gt; 60/190,554

&lt;151&gt; 2000-03-20

&lt;150&gt; 60/210,734

&lt;151&gt; 2000-06-12

&lt;150&gt; 60/255,147

&lt;151&gt; 2000-12-14

&lt;160&gt; 2

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 1579

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 1

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&lt;210&gt; 2

&lt;211&gt; 451

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 2

```

Met Glu Ser Ser Pro Ile Pro Gln Ser Ser Gly Asn Ser Ser Thr Leu
  1              5              10              15

```

```

Gly Arg Val Pro Gln Thr Pro Gly Pro Ser Thr Ala Ser Gly Val Pro
          20              25              30

```

```

Glu Val Gly Leu Arg Asp Val Ala Ser Glu Ser Val Ala Leu Phe Phe
          35              40              45

```

```

Met Leu Leu Leu Asp Leu Thr Ala Val Ala Gly Asn Ala Ala Val Met
          50              55              60

```

```

Ala Val Ile Ala Lys Thr Pro Ala Leu Arg Lys Phe Val Phe Val Phe
          65              70              75              80

```

```

His Leu Cys Leu Val Asp Leu Leu Ala Ala Leu Thr Leu Met Pro Leu
          85              90              95

```

```

Ala Met Leu Ser Ser Ser Ala Leu Phe Asp His Ala Leu Phe Gly Glu
          100              105              110

```

```

Val Ala Cys Arg Leu Tyr Leu Phe Leu Ser Val Cys Phe Val Ser Leu
          115              120              125

```

```

Ala Ile Leu Ser Val Ser Ala Ile Asn Val Glu Arg Tyr Tyr Tyr Val
          130              135              140

```

```

Val His Pro Met Arg Tyr Glu Val Arg Met Thr Leu Gly Leu Val Ala
          145              150              155              160

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Ser Val Leu Val Gly Val Trp Val Lys Ala Leu Ala Met Ala Ser Val	165	170	175
Pro Val Leu Gly Arg Val Ser Trp Glu Glu Gly Ala Pro Ser Val Pro	180	185	190
Pro Gly Cys Ser Leu Gln Trp Ser His Ser Ala Tyr Cys Gln Leu Phe	195	200	205
Val Val Val Phe Ala Val Leu Tyr Phe Leu Leu Pro Leu Leu Leu Ile	210	215	220
Leu Val Val Tyr Cys Ser Met Phe Arg Val Ala Arg Val Ala Ala Met	225	230	235
Gln His Gly Pro Leu Pro Thr Trp Met Glu Thr Pro Arg Gln Arg Ser	245	250	255
Glu Ser Leu Ser Ser Arg Ser Thr Met Val Thr Ser Ser Gly Ala Pro	260	265	270
Gln Thr Thr Pro His Arg Thr Phe Gly Gly Gly Lys Ala Ala Val Val	275	280	285
Leu Leu Ala Val Gly Gly Gln Phe Leu Leu Cys Trp Leu Pro Tyr Phe	290	295	300
Ser Phe His Leu Tyr Val Ala Leu Ser Ala Gln Pro Ile Ser Thr Gly	305	310	315
Gln Val Glu Ser Val Val Thr Trp Ile Gly Tyr Phe Cys Phe Thr Ser	325	330	335
Asn Pro Phe Phe Tyr Gly Cys Leu Asn Arg Gln Ile Arg Gly Glu Leu	340	345	350
Ser Lys Gln Phe Val Cys Phe Phe Lys Pro Ala Pro Glu Glu Glu Leu	355	360	365
Arg Leu Pro Ser Arg Glu Gly Ser Ile Glu Glu Asn Phe Leu Gln Phe	370	375	380
Leu Gln Gly Thr Gly Cys Pro Ser Glu Ser Trp Val Ser Arg Pro Leu	385	390	395
Pro Ser Pro Lys Gln Glu Pro Pro Ala Val Asp Phe Arg Ile Pro Gly	405	410	415

Gln Ile Ala Glu Glu Thr Ser Glu Phe Leu Glu Gln Gln Leu Thr Ser

420

425

430

Asp Ile Ile Met Ser Asp Ser Tyr Leu Arg Pro Ala Ala Ser Pro Arg

435

440

445

Leu Glu Ser

450